DIRECT DEMONSTRATION OF THETA-POSITIVE ANTIGEN-BINDING CELLS, WITH ANTIGEN-INDUCED MOVEMENT OF THYMUS-DEPENDENT CELL RECEPTORS

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Our understanding of the immune response has been advanced considerably by the realization that there are two major populations of lymphocytes, one thymus-dependent (T cells)[1] and one thymus-independent (B cells), with different origins, functions, and properties (1, 2). Although at present the two types of lymphocytes are morphologically indistinguishable, in mice they can be distinguished by surface antigenic markers, such as the alloantigen θ on T cells (3, 4) and the heteroantigen MBLA (mouse-specific B lymphocyte antigen) on B cells (5, 6).

There is abundant evidence that T cells can respond specifically to immunization, in that they can carry immunological memory and become immunologically tolerant (reviewed in 2). Thus the conclusion that T lymphocytes possess antigen-specific receptors seems as inescapable as it is in the B cell, where increasing evidence suggests that the antigen-receptor is immunoglobulin (Ig), with its combining site identical to that of the antibody the cells' progeny will later produce (7, 8).

However, attempts to demonstrate Ig on the surface of T cells have given remarkably variable results and the chemical nature of the T cell receptor is still in dispute. A separate, but perhaps related, problem has been the difficulty in demonstrating T cells binding antigen to their surface (9) or adhering to antigen-coated beads (10), which in turn has made it difficult to study T cell receptors.

Thus far, attempts to identify antigen-binding T cells have been indirect, depending largely on demonstrating antigen-binding cells in thymus (11, 12) or on showing that a proportion of antigen-binding peripheral lymphocytes can be eliminated by anti-θ serum and complement (11, 13–15). This paper reports the first direct demonstration of antigen-binding T cells. Fluorescein-conjugated anti-θ antibody has been used to identify individual T cells binding sheep erythrocytes (SRBC) to their surface and to show that under appropriate conditions the antigen induces a redistribution of the T cell receptors in a manner similar to that previously described for B cells (16). In addition, anti-Ig antibody is shown to block the ability of the T cells to bind SRBC.

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[1] *Abbreviations used in this paper:* Anti-θAKR-F, anti-θAKR conjugated to fluorescein; B cells, thymus-independent cells; Ig, immunoglobulin; RFC, rosette-forming cells; SRBC, sheep erythrocytes; T cells, thymus-dependent cells.

Materials and Methods

Animals.—Male and female A and CBA mice 3–6 mo of age and female AKR mice 2–5 mo of age, from the breeding colony of the National Institute for Medical Research, were used. A-strain mice congenic for θAKR(A/θ)AKR) were raised from breeding pairs kindly supplied by Dr. E. A. Boyse. Sheep erythrocytes (2×10^8 in 0.1 ml intraperitoneally) were administered 5 days before sacrifice unless otherwise noted.

Antiserum Preparation.—Anti-θAKR was prepared by injecting CBA mice with 10^7 AKR thymocytes intraperitoneally each week for 6–9 wk before bleeding (17). The titer (end of cytotoxic plateau) of the pooled serum was 1/4096 as measured by ^{51}Cr cytotoxicity testing with absorbed hamster complement (4) against (CBA × AKR) F_1 thymocytes. The serum was heat inactivated, the immunoglobulin precipitated with 40% saturated ammonium sulfate, and the redissolved precipitate conjugated with fluorescein isothiocyanate as previously described (18). The final protein concentration was 10 mg/ml and OD 485 nm/OD 280 nm was 0.8.

The preparation of rabbit IgG anti-mouse Ig has been previously described[2]: 3.4% of this rabbit IgG was specifically precipitable by diethylaminoethyl (DEAE)-cellulose-purified CBA mouse IgG. Coupling of mouse IgG to 4B Sepharose was performed by the cyanogen bromide method (19).

Assays.—Dye exclusion and ^{51}Cr cytotoxicity testing were performed as previously described using absorbed hamster complement with thymocytes and absorbed guinea pig complement with spleen cells (20).

Spleen cells were suspended, incubated on cotton wool by the method of Hogg and Greaves (21) to remove macrophages, granulocytes, and debris, and washed two times in balanced salt solution with a final wash in Veronal-buffered saline. Details are given elsewhere.[2] Thymus cells were prepared similarly but without cotton wool incubation. Unless specified otherwise, cells were then incubated at 25×10^6/ml with various concentrations of anti-θAKR fluorescein (anti-θAKR-F) for 20 min at 23°C, then washed two times with cold buffer. 40 μl of 25×10^6/ml cells were then mixed with 20 μl of 2% vol/vol sheep erythrocytes and centrifuged 10 min at 50 *g* in the cold (ratio of SRBC/spleen cells, 8:1). After remaining as a pellet for 10 min, the rosettes were resuspended gently in about 20 μl and kept in an ice bath. Rosette suspensions were never centrifuged at more than 50 *g*. About 10 μl of cell suspension was sealed by paraffin under a cover slip on an acid-alcohol-cleaned slide and systematically searched for rosettes under oil immersion at 1000 × with a Vickers M41 Photoplan microscope modified so as to permit rapid sequential viewing of the same field under blue-violet incident illumination or transmitted illumination with phase contrast. Such an arrangement was essential in order that rosettes could be distinguished clearly from cell clumps and that fluorescence could be localized with certainty to the surface of the central lymphocyte. Two 3-mm BG12 exciter filters and one Ilford 107 barrier filter were employed with blue-violet illumination. Slides were prepared immediately before scoring; 45 min to 1 h was required to complete a slide, a typical slide yielding 30–60 rosettes. As described previously,[2] all lymphocytes bearing five or more bound SRBC were counted as rosettes unless they appeared dead or damaged (swollen cytoplasm, vacuoles, clearly demarcated nucleus, or diffuse internal fluorescence) or were so closely associated with other lymphocytes that fluorescence, if present, could not have been unambiguously assessed. Where more than one preparation was being scored, slides were examined from all preparations in a regular rotation so as to minimize any effects of time. Cells survived well in ice as long as 8 h after staining, but were never kept longer. In most experiments, rosettes were classified according to the number of bound SRBC: 5, 6–7, or ≥8, and the latter group further subdivided according to whether an unobstructed view of most of the surface of the

[2] Ashman, R. F. Concurrent movement of antigen receptors and surface immunoglobulin on antigen-binding cells. Submitted for publication. The proportion of lymphocyte circumference covered by bound SRBC is > ¾ for "rings," ½–¾ for "horseshoes," and ≤ ½ for "caps."

rosette-forming cell was afforded (subgroup A) or not (subgroup B). The B subgroup had many more bound SRBC's on the average than the A subgroup.

To test for inhibition of rosette formation, cells were incubated with anti-immunoglobulin and anti-θ without complement at 0°C for 1 h immediately before addition of SRBC (13, 22). To test the specificity of this inhibition, similar incubations were performed with anti-Ig which had been absorbed with mouse Ig-Sepharose or unmodified Sepharose and with mouse IgG present along with anti-Ig.

To test whether cells could form rosettes by means of anti-erythrocyte antibodies acquired from the serum, mice were injected with 2×10^8 human RBC in addition to the usual dose of 2×10^8 SRBC and the rosette-forming cells present on day 5 were examined for the presence of cells binding both species of erythrocytes, using an 8:1 ratio of each species of RBC to spleen cells.

Receptor movement was induced by incubation of rosette suspensions at 37°C in balanced salt solution. At each time point, sample tubes were transferred to an ice bath and immediately examined. Rosettes were classified by the distribution of SRBC as caps, horseshoes, or rings according to the criteria described previously.[2] Controls included (*a*) incubating spleen cells alone at 37°C and forming rosettes when they were transferred to the ice bath; (*b*) incubating rosette suspensions in the ice bath only; and (*c*) incubating rosettes for 30 min at 37°C in various concentrations of sodium azide. Rosette suspensions incubated at 37°C for 30 min to produce maximal receptor movement were then cooled and 0.4 mg/ml sodium azide was added to prevent further capping. Then the suspensions were stained with anti-θAKR-F as previously described.

Standard errors of percentages are not based on multiple determinations but on an assumed normal distribution of observations about the true value. Since they cannot take into account measurement errors or biological variation, they must be considered minimum estimates of variance more useful for determining the absence of a significant difference than its presence. In all tables and figures the confidence intervals quoted represent one standard error.

RESULTS

The experiments showed that the anti-θAKR-F was monospecific for θ and could be used to detect θ on peripheral T cells including T-rosette-forming cells (T-RFC). It was also found that anti-Ig prevented both T and B cells from binding SRBC and that the receptors of T and B cells could migrate toward one pole of the cell under the influence of bound SRBC.

Specificity of Anti-θAKR Serum.—Any unabsorbed CBA anti-AKR-thymocyte serum might contain, in addition to anti-θAKR, a variety of autoantibodies (23–25); antibodies to LyA_2 (26), LyC_1 (27), and other undefined lymphocyte alloantigens (28); and antibodies to Ig allotypic determinants (29). It should not contain anti-*H-2* activity since CBA and AKR mice are both *H-2k*.

A number of experiments were done to establish the anti-θ specificity of the observed fluorescence and cytotoxicity. As Fig. 4 shows, about 37% of RFC and non-RFC from spleen 5 days after immunization showed surface fluorescence after treatment with anti-θAKR-F. A single absorption with CBA brain did not significantly reduce the activity either for RFC or non-RFC (compare Figs. 1 *a* and 4), although the absorption entailed a dilution of almost ½; nor did unabsorbed antiserum stain CBA spleen cells or RFC (Fig. 1 *b*), ruling out a significant contribution from autoantibody. Yet one absorption with AKR brain removed almost all the staining activity (Fig. 1 *a*), excluding an important con-

tribution from anti-Ly antibodies as Ly antigens do not occur in brain (26). The most convincing evidence that only anti-θAKR activity was being detected was the demonstration that when a congenic pair of strains were studied, which were genetically identical except at the θ locus, the one with θAKR(A/θAKR) stained and the one with θC3H(A) did not (Fig. 1 *b*). Fig. 2 makes the same point with reference to thymus and shows that by cytotoxicity and direct immunofluorescence under the conditions described here, our antiserum appears to detect θAKR and nothing else. The greatest dilution still giving plateau cy-

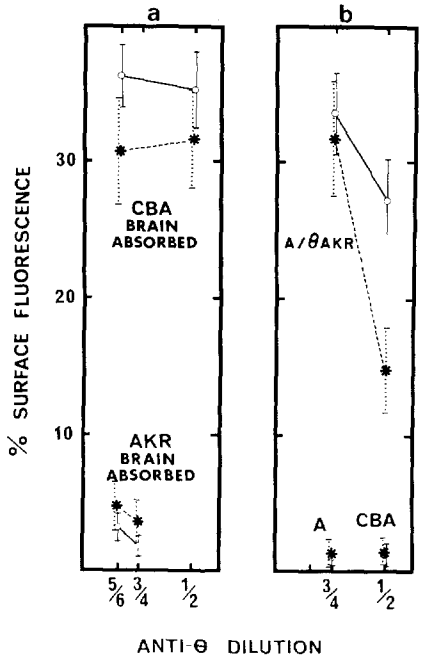

FIG. 1. Specificity of anti-θAKR staining of rosette-forming cells. Percent surface fluorescence refers to the proportion of RFC or non-RFC showing surface fluorescence. Brackets denote one standard error. ✳ - - - ✳, rosette-forming cells; ○—○, other lymphocytes. (*a*) Anti-θAKR-F was incubated for 40 min at 23°C with an equal volume of washed CBA or AKR brain homogenate, entailing a dilution not greater than ½. (*b*) Spleen rosette suspensions of strains A, A/θAKR, and CBA mice were exposed to unabsorbed anti-θAKR-F at the concentrations noted for 20 min at 23°C.

totoxicity was $\frac{1}{128}$ for AKR spleen cells and $\frac{1}{4096}$ for AKR thumus. Fluorescence titers were significantly lower (see below).

Fluorescent Staining of the Spleen and Thymus Cells and Spleen Rosette Suspensions with Anti-θAKR.—Anti-θAKR-F gave diffuse, smooth ring staining (without significant patching or capping) of >95% of thymocytes (Fig. 3) and 30–40% of spleen lymphocytes, regardless of the temperature at which the staining was carried out. As expected, the fluorescence of spleen cells was significantly less intense than that of thymocytes (30, 31). In order to achieve the

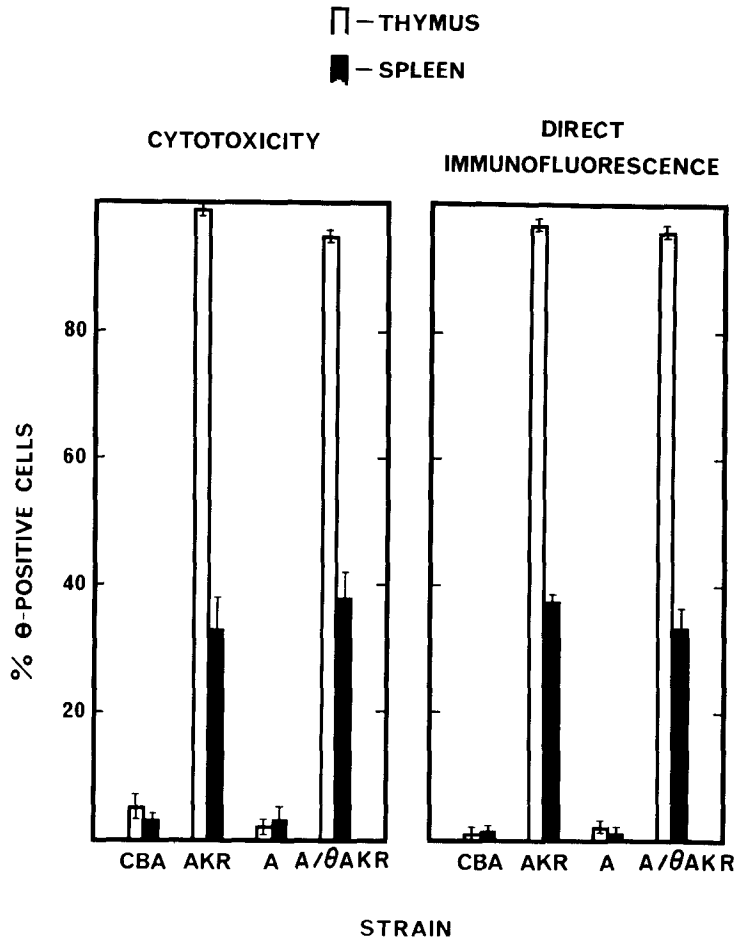

FIG. 2. Specificity of anti-θAKR investigated by direct immunofluorescence and cytotoxicity against thymus and spleen. Percent surface fluorescence and brackets have the same meaning as in Fig. 1. Solid bars represent plateau values for cotton-filtered spleen cells; open bars represent plateau values for unfiltered thymus. Direct immunofluorescence was performed by incubation of cells at 25×10^6/ml with ¾ or less dilution of anti-θAKR-fluorescein for 20 min at 23°C. Cytotoxicity was performed by trypan-blue exclusion employing hamster complement with thymus cells and guinea pig complement with spleen cells. All percentages were obtained with concentrations on the cytotoxic or immunofluorescence plateaus derived for AKR spleen cells.

maximal percent of fluorescence-positive non-rosette-forming spleen cells (non-RFC), the anti-θAKR-F had to be used at a dilution of ⅛ or less (Fig. 4). In contrast, rosette-forming cells (RFC) required at least ½ anti-θAKR-F for the maximum number to stain (Fig. 4).

Numbers of rosettes scored varied from 50 at 1/64 dilution and 134 at 1/32 to 379 at ½; numbers of non-RFC's scored varied from 223 at ⅛ to 811 at 5/6.

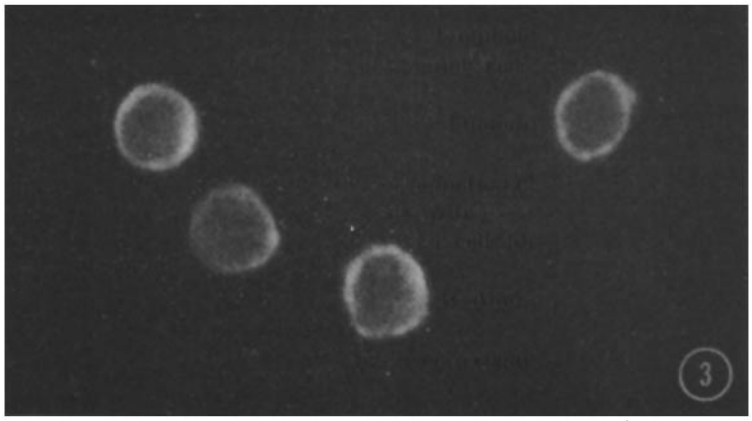

FIG. 3. AKR thymocytes stained with anti-θAKR-fluorescein at 23°C showing smooth ring fluorescence. × 2,450.

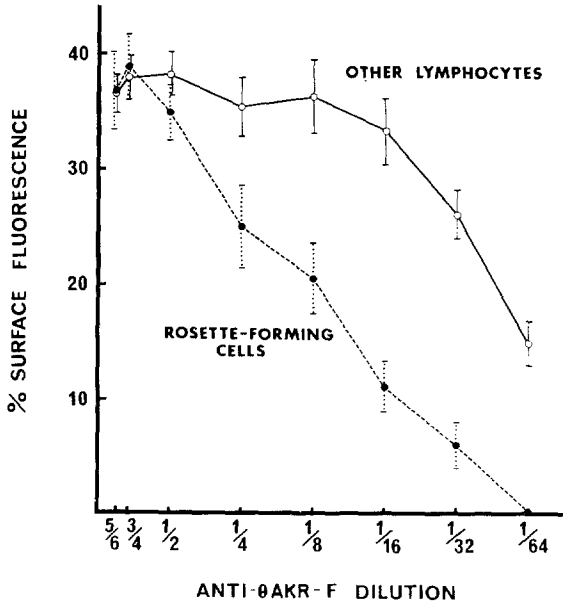

FIG. 4. Fluorescent staining of AKR spleen lymphocytes (normal and rosette-forming) with anti-θAKR-fluorescein. Dilutions of anti-θAKR-F plotted are those obtaining during a 20-min incubation of 25 × 10^6/ml spleen lymphocytes in 40 μl at 23°C. Rosettes were formed after two washes in the cold, and remained at 0°C until they were scored. RFC and non-FRC were scored on the same slide simultaneously. Percent surface fluorescence refers to the proportion of cells showing surface fluorescence. Brackets represent one standard error above and below the mean.

Three experiments were done at $\frac{5}{6}$ and $\frac{1}{2}$, two at $\frac{3}{4}$, and one experiment at each of the other dilutions.

The eight experiments performed at dilutions $\frac{1}{2}$ to $\frac{5}{6}$ gave percentages of θ^+ RFC ranging between 32 and 39%. The dilution curves (Fig. 4) show that the

fluorescence titer of the anti-θAKR-F was five to eight times higher when tested against spleen non-RFC than when tested against RFC. If one excluded those rosettes where much of the RFC surface was obscured by SRBC (≥ 8B, Fig. 5), there is still a consistent factor of 4 separating the two curves. Even at anti-θAKR-F concentrations of ½ to 5/6, the fluorescence of RFC was obviously less intense than that on non-RFC although an occasional bright RFC was seen.

In a single experiment with unimmunized spleen using ½ dilution of anti-θAKR-F, 45 ± 6% of RFC and 38 ± 2% of non-RFC were θ^+. 18 days after

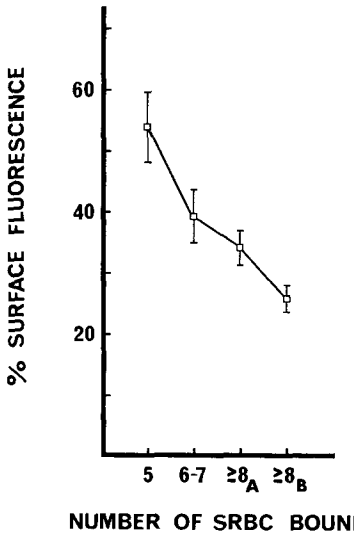

FIG. 5. Relationship of θ to number of SRBC bound. Anti-θ data is pooled from all experiments where concentrations of ½ anti-θ or greater were used, i.e., where staining is near maximal. Percent surface fluorescence refers to the proportion of RFC in each category showing surface fluorescence. Rosettes are classified according to the number of bound SRBC. All those with eight or more SRBC are grouped according to whether the distribution of SRBC permits an unobstructed view of most of the lymphocyte surface ($\geq 8_A$) or not ($\geq 8_B$).

immunization 24 ± 4% of RFC and 36 ± 3% of non-RFC were θ^+. When the data with 5-day immune rosettes obtained with dilutions between ½ and 5/6 was reanalyzed with respect to the number of bound SRBC (Fig. 5), a striking tendency of the θ^+ RFC to bind fewer SRBC than the θ^- RFC emerged.

Inhibition of AKR Rosettes by Anti-Ig But Not by Anti-θAKR.—Rabbit antibody to mouse Ig inhibited 99.5% of rosettes in AKR mice (Fig. 6). If the anti-Ig in this preparation is removed on a mouse-IgG-4B-Sepharose column and the original protein concentration restored, no inhibitory activity remained. Absorption with 4B Sepharose alone did not alter the activity.

The presence of mouse IgG during the preincubation of cells with anti-IgG completely prevented the inhibition of rosette formation at IgG/anti-IgG ratios of 2.5 to 60, showing that immune complexes in antigen excess possessed no de-

tectable inhibitory activity (Fig. 7). Since 37% of the AKR rosettes were θ^+, both T and B rosettes were apparently inhibitable by anti-Ig.

In the cold and in the absence of complement, anti-θAKR could not prevent rosette formation (Fig. 6), nor could it dissociate rosettes once formed (Table I). Table I shows an experiment which duplicated the procedure used to stain rosettes and shows that the sample of rosettes actually examined for fluorescence was about 89% of the original number. The small loss was probably attributable to centrifuging rosettes through protein, since it occurred with fetal

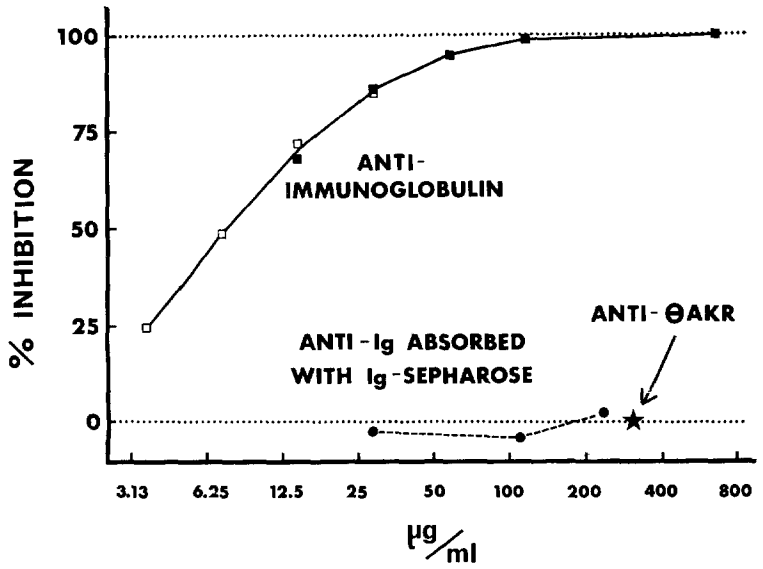

FIG. 6. Inhibition of AKR rosette formation with anti-Ig. 200 μl spleen cells at 25 $\times$ 10^6/ml are incubated 1 h at 0°C with the designated concentrations of rabbit IgG anti-mouse IgG (3.4% of which was specifically precipitable by mouse IgG) with (●) or without (□, ■) absorption on Ig-Sepharose column. Closed and open squares refer to two overlapping experiments performed on different days. The concentration of anti-θAKR noted refers to the 40% saturated ammonium sulfate fraction of heat-inactivated serum.

calf serum at the same concentrations. It was not eliminated by merely spinning longer.

Double-Binding Experiment.—5 days after immunization with 2 $\times$ 10^8 human and 2 $\times$ 10^8 sheep RBC, there were 1.3 human and 4.9 sheep rosettes per 10^3 spleen cells, about 40% of the rosette counts obtained if mice received 2 $\times$ 10^8 of either species of red cells alone. Of the 139 human and 520 sheep rosettes examined, only one rosette was suspected of binding more than one species of RBC. Thus neither T nor B RFC bound more than one type of RBC.

Receptor Movement.—Fig. 8 shows that incubating rosettes at 37°C in balanced salt solution induced movement of receptors on a proportion of cells. This "capping" of receptors was antigen induced because, as Fig. 8 shows, it

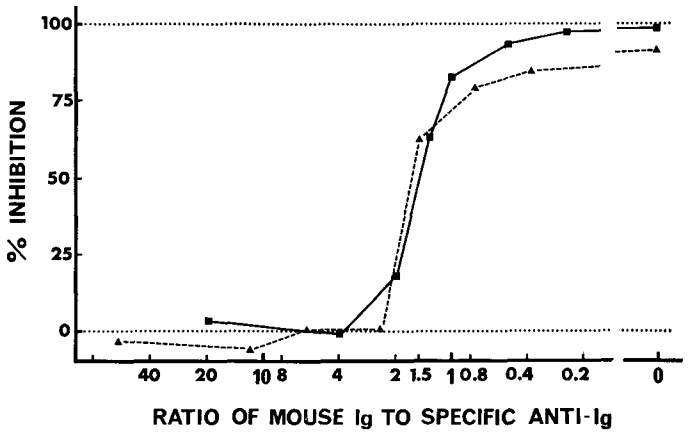

FIG. 7. Blocking of anti-Ig rosette inhibition by Ig. The two curves represent concurrent experiments performed using 164 μg/ml (■—■) and 55 μg/ml (▲ - - - ▲) of rabbit IgG anti-mouse IgG. 200 μl of immune spleen cells at 25 × 10^6/ml were incubated 1 h at 0°C with anti-Ig and mouse IgG together; then, without washing, SRBC rosettes were formed. Since the molecular weight of rabbit and mouse IgG's are similar the mass and molar ratios are alike.

TABLE I

*Rosette Loss During Staining Procedure**

Steps	Tube				
	Experiment a		Experiment b		
	1	2	1	2	3
20-min incubation	Medium alone at 0°C	23°C with 5/6 dilution anti-θAKR	Medium alone at 0°C	23°C with 3/4 dilution fetal calf serum	23°C with 3/4 dilution anti-θAKR
2 washes at 50 *g* 4°C	—	+	—	+	+
Rosettes per hemacytometer	235 241	216 206	216 212	196 183	179 190
Percent of control	100%	89%	100%	89%	86%

* Unconjugated anti-θAKR (without added complement) was used at the same concentration employed in the staining procedure, which the experiment was designed to mimic. Both fetal calf serum and anti-θAKR were heat inactivated.

did not occur in the absence of antigen. Like anti-Ig-induced capping (16) it failed to occur in the presence of antigen at 0°C or in the presence of antigen at 37°C in 0.4 mg/ml sodium azide. Smaller concentrations of azide produced less complete inhibition of capping, and 1.0 mg/ml produced no better inhibition than 0.4 mg/ml. By incubating rosettes at 37°C for 30 min, halting the

receptor movement with 0.4 mg/ml azide, and staining with anti-θAKR-F at $\frac{3}{4}$ dilution, one could show (Table II, top section) that the percent of rosettes with SRBC distributed as a cap or horseshoe which were θ^+ (44 ± 7%) was not significantly different from the percent of ring rosettes which were θ^+ (35 ± 4%), and that 21 of the 23 θ^+ caps or horseshoes had their θ distributed in an unbroken ring. When azide is present during the 37°C incubation, receptor movement was minimal, but the 4 θ^+ rosettes which had shown receptor movement all had ring θ. Likewise, at anti-θAKR-F dilutions $\frac{1}{2}$ to $\frac{5}{6}$ in Fig. 4, with

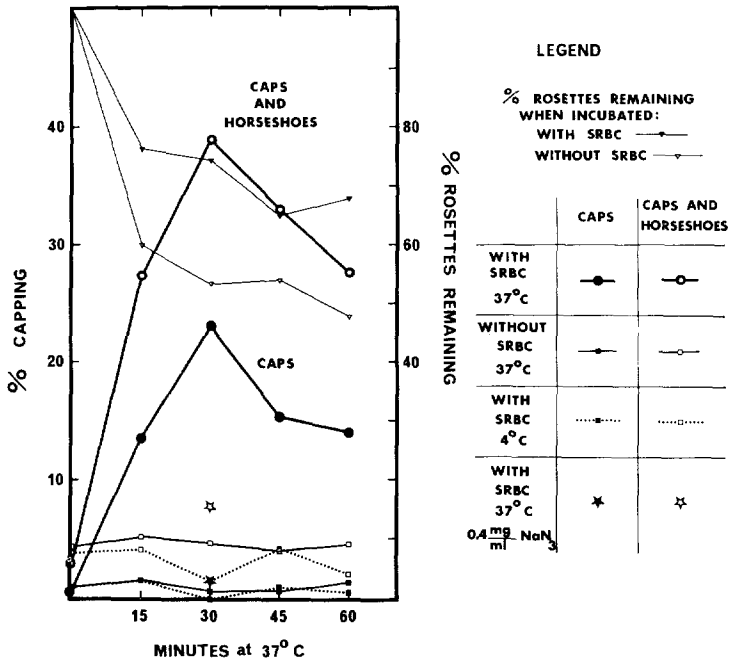

FIG. 8. Antigen-induced capping.

rosettes stored in ice, only $^{36}\!/_{1120}$ rosettes were θ^+ caps or horseshoes, but 30 of these had symmetrical θ. Thus it is clear that receptors can move in the membrane without affecting the distributions of θ antigen, and the evidence suggests that T cell receptors "cap" at least as readily as B cell receptors.

DISCUSSION

Theta (θ) has proved a useful marker for T cells in mice. Heretofore, θ-bearing cells have been detected by cytotoxicity testing or by indirect immunofluorescence using fluorescein-conjugated anti-mouse Ig sera to demonstrate the bound anti-θ alloantibody (31). Unfortunately, the former method cannot be used to identify living T cells, and the latter method, when applied to T cells in the peripheral lymphoid tissues, is complicated by the fact that B cells also

TABLE II

Relationship of Distribution of Receptors to Distribution of θ

Conditions of rosette formation and incubation	No. of rosettes scored	θ*	Pattern of distribution‡ of SRBC	Pattern of distribution‡ of θ by surface fluorescence
37°C 30 min	167	θ+ 63	23 caps and horseshoes	2 caps and horseshoes
				21 rings
			40 rings	3 caps and horseshoes
				37 rings
		θ− 104	29 caps and horseshoes	
			75 rings	
37°C 30 min in 0.4 mg/ml sodium azide	107	θ+ 38	4 caps and horseshoes	0 caps and horseshoes
				4 rings
			34 rings	2 caps and horseshoes
				32 rings
		θ− 69	2 caps and horseshoes	
			67 rings	

* As determined by staining with anti-θAKR-F.

‡ Classification scheme depicted in Fig. 1.[2] Proportion of lymphocyte circumference covered by bound SRBC is > ¾ for rings, ½–¾ for horseshoes, and ≤ ½ for caps.

fluoresce because of their abundant surface Ig (31). Thus in order to demonstrate live peripheral T cells, a fluorescein-conjugated anti-θ suitable for direct immunofluorescence was required. Previously it was found that a fluoresceinated anti-θC3H serum stained thymocytes faintly; but peripheral T cells, which are known to bear much less θ than thymocytes (30), failed to stain (31). However, when we found that, in general, anti-θAKR sera had much higher cytotoxicity titers than anti-θC3H sera, we were encouraged to try again with fluorescein-conjugated anti-θAKR antibody (anti-θAKR-F).

As Fig. 2 shows, > 95% of AKR thymocytes and 30–40% of spleen cells were stained directly by anti-θAKR-F; the same proportion were killed in cytotoxic testing. In both assays, increasing concentrations of antibody produced plateaus. Thus, although the titer against spleen cells was eight times higher by cytotoxicity, the immunofluorescence titer was sufficient to detect all θ^+ cells in spleen.

The specificity of the antiserum for θAKR in cytotoxicity and immunofluorescence tests was clearly established, despite the probable presence of a variety of contaminating antibodies to non-θ determinants. Most convincing was the demonstration of cytotoxic and fluorescence activity against A/θAKR cells (including rosette-forming cells), with negligible activity against A (θC3H) cells (Figs. 1 *b* and 2). These two congenic lines presumably differ only at the θ locus. Because the anti-θAKR-F could be used to specifically stain θ^+ peripheral T lymphocytes, it provided the first opportunity to observe living T cells binding antigen and to study the behavior of their receptors.

Although there is no doubt that T cells have specific receptors for antigen (32), attempts to demonstrate antigen binding by T cells have frequently been unsuccessful (9, 33). In general, methods involving the recognition of cell-surface antigens by T cells have been more successful (10, 34) than methods involving soluble antigen. However, even the demonstration of T cells binding SRBC has not been achieved by every laboratory which has attempted it (9). Most of these failures are probably related to the methods used to prepare rosettes, for T-RFC are much more fragile than B-RFC.[3] It is not clear whether the fragility of T cell antigen binding is related to receptor affinity, density, turnover, or shedding on contact with antigen, or some nonreceptor property of T cell membranes. Failure to detect T-RFC could also result from a weak anti-θ antiserum; Fig. 4 shows that if our anti-θAKR had had $\frac{1}{32}$ as much antibody, we might well have demonstrated θ on 25–30% of spleen cells, but remained unconvinced that the 5% of faintly stained rosettes were meaningful. The debate concerning the existence of T-RFC is far from trivial, since so much of our information about T cell receptors derives from the rosette technique.

The results reported here should put an end to that debate, although the

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[3] Elliott, B. E., and J. S. Haskill, manuscript in preparation; unpublished observations of N. Hogg and J. F. Bach, personal communication.

immunological significance of T-RFC and the nature of the receptor binding the SRBC no doubt will continue to be controversial. Our failure to find T cells binding both human and sheep erythrocytes in the spleen of doubly immunized mice suggests that the binding is immunologically specific and not related to antibody absorbed nonspecifically from the serum. The demonstration that a polyspecific anti-Ig serum can prevent T cells as well as B cells from binding SRBC, and that this inbibitory activity is abolished by removing the relevant antibodies with Ig-Sepharose or by adding soluble mouse Ig, certainly suggests that the T cell receptors for SRBC are Ig (Figs. 6 and 7). Furthermore, the fraction of rosettes specifically dissociated by anti-Ig (but not by Ig-Sepharose-absorbed anti-Ig) must include the T-RFC, since 90% of the remaining rosettes have surface Ig demonstrable by fluorescence.[2] A similar conclusion has been reached in CBA mice using anti-θC3H plus complement to detect T-RFC, a study using class-specific antisera which was particularly detailed and rigorously controlled (35). Our results also confirm other previous observations on T-RFC based on the cytotoxicity of anti-θC3H. (*a*) The proportion of T-RFC decreasing during the first few weeks after immunization roughly agrees with previous observations (11, 28), though our figures are lower than the estimates of Bach, Muller, and Dardenne (11) and totally different from those of Schlesinger (14). (*b*) T-RFC bind fewer SRBC on average than B-RFC (Fig. 5) (21, 36). (*c*) T-RFC in spleens of 5-day immunized mice express less θ than other spleen T cells (28).

Recently it has been suggested that the redistribution of B cell surface Ig receptors ("patch" and "cap" formation with or without pinocytosis) which can be induced by divalent anti-Ig or multivalent antigens, may play a role in the triggering of B cells by antigen and perhaps in some forms of tolerance induction (16, 37). Until now, the difficulty in directly demonstrating receptors on T cells or antigen binding by T cells has made it impossible to study the behavior of T cell receptors upon interaction with antibody or antigen. In this study we have found that T cell receptors cap under the influence of bound antigen (SRBC) at least as readily as those of B cells (Table II).

Even on T cells whose SRBC-receptors have capped, the anti-θAKR-F remains in a diffuse ring; this shows that θ and the receptors can move independently in the membrane. This conclusion is reinforced by the failure of anti-θ in the absence of complement to block SRBC binding to any cells, including T cells (Table I and Fig. 6).

In another study it was shown that receptors for SRBC and Ig move together on the surface of B cells, presumably because the Ig molecules are the receptors.[2, 4] Since we have not been able to directly demonstrate Ig on the surface of T cells, this type of experiment could not be done with T-RFC.

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[4] Ashman, R. F. Lymphocyte receptor movements induced by antigen binding. Submitted for publication.

Anti-θAKR-F gave diffuse, smooth ring staining of thymus cells and spleen T cells (Fig. 3) even when the staining was done at 37°C. This suggests that anti-θ itself does not induce patching or capping, although it has been previously shown that subsequent addition of anti-mouse Ig can induce these changes in the distribution of θ on thymus cells (16), presumably by cross-linking the anti-θ molecules on the cell surface. This same phenomenon has been found with anti-*H-2* (16, 38) and anti-TL sera (39), and contrasts with the ability of anti-Ig to directly induce patching and capping of surface Ig on B cells (16). Whether this difference is related to the valences of the antibodies and surface antigens, the density of surface antigenic determinants, the strength of association between these determinants and other membrane elements, or other membrane properties remains unknown.

SUMMARY

Anti-θAKR antibody conjugated to fluorescein has been used in direct immunofluorescence tests to identify spleen θ^+ (T) sheep erythrocyte rosette-forming cells in AKR mice. Specificity studies involving A and cogenic A/θAKR mice clearly demonstrated that the cell surface fluorescence and cytotoxicity produced by the antiserum is directed solely toward the θAKR alloantigen. Approximately $\frac{3}{8}$ of rosette-forming and non-rosette-forming spleen cells were found to be θ^+. The tendency for T cells to bind less antigen and the tendency for antigen-binding T cells to bear less θ than other spleen T cells, first suggested by other studies involving rosette-elimination by anti-θC3H plus complement, were confirmed by direct immunofluorescence. All AKR rosettes are specifically inhibitable by anti-immunoglobulin, including T rosettes. Antigen-induced redistribution of T cell receptors, analogous to that previously described for B cell receptors (16), occurs as readily in θ^+RFC as in θ^- RFC, without altering the symmetrical ring distribution of θAKR antigen.

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REFERENCES

1. Antigen-Sensitive Cells. Their Source and Differentiation. 1969. *Transplant. Rev.* **1.**
2. Greaves, M. F., J. J. Owen, and M. C. Raff. 1973. T and B lymphocytes: origins, properties and roles in immune responses. *Excerpta Med. Int. Congr. Ser.* In press.
3. Schlesinger, M., and I. Yron. 1969. Antigenic changes in lymph node cells after administration of antiserum to thymus cells. *Science* (*Wash. D.C.*). **164:**1412.
4. Raff, M. C. 1969. Theta isoantigen as a marker of thymus-derived lymphocytes in mice. *Nature* (*Lond.*). **224:**378.
5. Raff, M. C., S. Nase, and N. A. Mitchison. 1971. Mouse-specific bone marrow-derived lymphocyte antigen (MBLA)—a marker for thymus independent lymphocytes. *Nature* (*Lond.*). **230:**50.

6. Niederhuber, J. E., and E. Möller. 1972. Antigenic markers on mouse lymphoid cells: the presence of MBLA on antibody-forming cells and antigen binding cells. *Cell. Immunol.* **3**:557.
7. Mäkela, O. 1970. Analogy between lymphocytes receptors and resulting humoral antibody. *Transplant. Rev.* **5**:3.
8. Cosenza, H. 1972. Anti-receptor antibodies. II. Specific inhibition of induction of an antibody response to a pneumococcal antigen. *Fed. Proc.* **31**:752. (Abstr.)
9. Hunter, P., A. Munro, and I. McConnell. 1972. Properties of educated T-cells for rosette formation and co-operation with B-cells. *Nat. New Biol.* **236**:52.
10. Wigzell, H. 1971. Cellular immunosorbents. *In* Progress in Immunology. B. Amos, editor. Academic Press Inc., New York. 1105.
11. Bach, J. F., J. Y. Muller, and M. Dardenne. 1970. *In vivo* specific antigen recognition by rosette-forming cells. *Nature (Lond.)*. **227**:1251.
12. Modabber, F., S. Morikawa, and A. H. Coons. 1970. Antigen-binding cells in normal mouse thymus. *Science (Wash. D.C.)*. **170**:1102.
13. Greaves, M. F., and E. Möller. 1970. Studies on antigen-binding cells. I. The origin of reactive cells. *Cell. Immunol.* **1**:372.
14. Schlesinger, M. 1970. Anti-θ antibodies for detecting thymus-dependent lymphocytes in the immune response of mice to SRBC. *Nature (Lond.)*. **226**:1254.
15. Roelants, G. E. 1972. Quantitation of antigen-specific T and B lymphocytes in mouse spleen. *Nat. New Biol.* **236**:252.
16. Taylor, R. B., W. P. H. Duffus, M. C. Raff, and S. de Petris. 1971. Redistribution and pinocytosis of lymphocyte surface Ig molecules induced by anti-immunoglobulin antibody. *Nat. New Biol.* **233**:225.
17. Reif, A. E., and J. M. V. Allen. 1966. Mouse thymic iso-antigens. *Nature (Lond.)*. **209**:521.
18. Raff, M. C., M. Sternberg, and R. B. Taylor. 1970. Immunoglobulin determinants on the surface of mouse lymphoid cells. *Nature (Lond.)*. **225**:553.
19. Porath, J., R. Axén, and S. Ernbach. 1967. Chemical coupling of proteins to agarose. *Nature (Lond.)*. **215**:1491.
20. Raff, M. C., and H. H. Wortis. 1970. Thymus dependence of θ-bearing cells in the peripheral lymphoid tissues of mice. *Immunology*. **18**:931.
21. Hogg, N. M., and M. F. Greaves. 1972. Ag-binding thymus-derived lymphocytes. I. rapid method for isolation of $\theta+$ antigen-stimulated cells. *Immunology*. **22**:959.
22. McConnell, I., A. Munro, B. W. Gurner, and R. R. A. Coombs. 1969. Studies on actively allergized cells. I. The cyto-dynamics and morphology of rosette-forming lymph node cells in mice and inhibition of rosette formation with antibody to mouse immunoglobulins. *Int. Arch. Allergy Appl. Immunol.* **35**:209.
23. Boyse, E. A., E. Bressler, C. Iritani, and M. Lardis. 1970. Cytotoxic γ-M autoantibody in mouse alloantisera. *Transplantation*. **9**:339.
24. Shirai, T., and R. C. Mellors. 1971. Natural thymocytic autoantibody and reactive antigen in New Zealand Black and other mice. *Proc. Natl. Acad. Sci. U.S.A.* **68**:1412.
25. Raff, M. C. 1971. Naturally-occurring autoantibodies to mouse thymocytes. *Isr. J. Med. Sci.* **7**:724.
26. Boyse, E. A., M. Miyazawa, T. Aoki, and L. J. Old. 1968. Ly-A and Ly-B: two

systems of lymphocyte isoantigens in the mouse. *Proc. R. Soc. Lond. B. Biol. Sci.* **170:175.**

27. Boyse, E. A., K. Itakura, E. Stockert, C. A. Iritani, and M. Miura. 1971. Ly-C: a third locus specifying alloantigens expressed only on thymocytes and lymphocytes. *Transplantation.* **11:**351.
28. Greaves, M. F., and M. C. Raff. 1971. Specificity of anti-θ sera in dytotoxicity and functional tests on T-lymphocytes. *Nat. New Biol.* **233:**239.
29. Baird, S., J. Santa, and I. Weissman. 1971. Anti-theta antisera may contain anti-allotype contamination. *Nat. New Biol.* **232:**56.
30. Aoki, T., U. Hammerling, E. de Harven, E. A. Boyse, and L. J. Old. 1969. Antigenic structure of cell surfaces; an immunoferritin study of the occurrence and topography of H-2, θ, and TL alloantigens on mouse cells. *J. Exp. Med.* **130:**979.
31. Raff, M. C. 1970. Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. *Immunology.* **19:**637.
32. Schlossman, S. 1972. Antigen recognition: the specificity of T-cells involved in the cellular immune response. *Transplant. Rev.* **10:**97.
33. Davie, J. M., A. S. Rosenthal, and W. E. Paul. 1971. Receptors on immunocompetent cells. III. Specificity and nature of receptors on dinitrophenylated guinea pig albumin ^{125}I-binding cells of immunized guinea pigs. *J. Exp. Med.* **134:**517.
34. Bankhurst, A. D., and J. D. Wilson. 1971. Detection of antigen-binding cells by combined rosette formation and autoradiography. *Nat. New Biol.* **234:**154.
35. Hogg, N. M., and M. F. Greaves. 1972. Antigen-binding thymus-derived lymphocytes. II. Nature of the immunoglobulin determinants. *Immunology.* **22:**967.
36. Basten, A., J. F. A. P. Miller, N. L. Warner, and J. Pye. 1971. Specific inactivation of thymus-derived (T) and non-thymus-derived (B) lymphocytes by ^{125}I-labelled antigen. *Nat. New Biol.* **231:**104.
37. Loor, F., L. Forni, and B. Pernis. 1972. The dynamic state of the lymphocyte membrane. Factors affecting the distribution and turnover of surface immunoglobulin. *Eur. J. Immunol.* **2:**203.
38. Davis, W. C. 1972. H-2 antigen on cell membranes: an explanation for the alteration of distribution by indirect labelling techniques. *Science (Wash. D.C.).* **175:** 1006.
39. Raff, M. C., and S. de Petris. 1972. Movement of lymphocyte surface antigens and receptors: the fluid nature of the lymphocyte plasma membrane and its immunological significance. *Fed. Proc.* In press.